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Human Papillomaviruses and Cervical Neoplasia: A Model for Carcinogenesis

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Summary: Human papillomaviruses are etiologic for cervical cancers and their pathologic precursors. As presented in this review, pathologic, epidemiologic, and molecular data all support a working model that accounts for the pathogenetic role of these viruses in cervical neoplasia. Diagnostic criteria and classification systems are discussed in light of this model. These insights point to a potential change in clinical screening systems for cervical cancer. In addition, vaccine trials for oncogenic HPVs have begun. In the long term, these trials may hold promise as truly specific preventive therapy for this common human cancer. Key Words: HPV—Papillomavirus—Cervix—Cancer—Pathogenesis—Screening.

Human papillomaviruses (HPVs) are the etiologic agents of cervical neoplasia. This simple statement is the product of more than two decades of work that has revealed the interplay of these common epitheliotropic viruses with their host cells. The pathologic classification of cervical neoplasia as well as the clinical management of these lesions increasingly reflects these biologic insights. I hope to review these concepts with emphasis on the mechanisms by which HPVs produce abnormal cervical morphology. This will be followed by a brief exploration of some applications of HPV-related technology.

HISTORICAL PERSPECTIVE

Historically, papillomaviruses have co-evolved with vertebrates. Virtually all vertebrate species have warts. Cutaneous warts have been described for thousands of years. In the beginning of this century, Ciuffo established the viral etiology of human warts (papillomas) by using cell-free extracts from wart tissue as an inoculum for man-to-man transmission experiments. In 1933, Shope described the first papillomavirus in cottontail rabbits. Subsequent experimentation in this system, including the use of coal tar as a tumor promoter, stimulated early concepts of cancer initiation and promotion, and produced one of the first examples of a human DNA tumor virus (1–3). The advent of electron microscopy brought the ultrastructural morphology of the papillomaviruses into focus. Clinical studies also revealed that different kinds of warts were more productive of virions than others. For example, plantar warts often had abundant viral particles, whereas genital warts had few (4–7). Since papillomaviruses are resistant to tissue culture and cannot be transmitted to laboratory animals, the characterization of this virus has been extremely difficult. Biochemical characterization and immunology carried out on viral proteins derived from direct extracts of warts provided early data suggesting that there was a single type of human papillomavirus, a view that was held through the 1960s (8). However, in the 1970s the revolution in modern biology permitted the molecular characterization of the papillomavirus family. Clones of the HPV genomes can be used to probe different pathologic processes, to establish the relationship of those lesions with HPVs. Analysis of the genomes isolated from these lesions reveals the plurality of HPV types based on DNA heterology (9,10). Papillomaviruses infect essentially all vertebrate species and induce primarily, albeit not exclusively, squamous epithelial neoplasias. In humans, more than 100 molecular types have been cloned, some two dozen of which are trophic for the anogenital tract (11,12). Anogenital HPV infections are the most com-

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mon sexually transmitted disease (13,14). The careful correlation of the clinical pathology of HPV-associated lesions with the molecular biology elucidated using the cloned viral DNAs as tools to dissect the virus-host interaction has been the key to our improved understanding of this common human cancer.

HPV VIROLOGY

The papillomaviruses have been traditionally classified as members of the papovavirus family. This family was named by taking the first two letters of the major genera: *pa*pilloma, *po*lyoma, and simian *va*cuolating viruses, respectively. All members of the family have a common structure; they are small, double-stranded DNA viruses that replicate in the nucleus and have icosahedral protein capsules that form nonenveloped virions. However, it has become apparent that the papillomaviruses are biologically distinct from SV40 and polyomavirus. Papillomaviruses have 55-nm rather than 40-nm diameter capsids; a reflection of that fact is that the approximately 8000-bp papillomavirus genome is 60% larger than the genome of polyomavirus. Moreover, the genome of polyomavirus will not cross-hybridize with papillomaviruses under low stringency conditions. The molecular organization of viral transcription is very different between HPV vs. polyoma; papillomaviruses transcribe all of their genes off one strand of the double-stranded genome. In contrast, SV40 and polyoma use a completely different bidirectional transcription strategy.

All papillomaviruses have a circular double-stranded DNA genome of approximately 8.0-kb complexity, encoding seven or eight early and two late genetic open reading frames (ORFs). Through gene splicing, the ORFs encode for all viral gene products. In addition, there is a noncoding region of approximately 1000 bp, often referred to as the upstream regulatory region (URR) or long control region, immediately upstream of the E6 ORF that contains the sequences regulating the expression of all ORFs (15). More than 20 messenger RNAs are expressed, normally in a highly differentiation- and cell-type-specific manner. ORFs E6 and E7 encode proteins that are capable of inducing cell proliferation and transformation. These are the only open reading frames that are conserved and expressed in *all* HPV-associated pathologies. The latter include the full spectrum from low-grade lesions with virtually no neoplastic potential to high-grade invasive cancers. The proteins encoded by E1 are involved in genome maintenance and replication. E2 encodes the major transregulatory proteins, which interact with the URR, having both positive and negative effects on transcription. The E4 ORF is the most abundantly transcribed message in a wart and is most highly expressed in differentiated cells. Some forms of E4 encode a protein, which binds to and disrupts the cytoplasmic keratin network producing what we recognize as a koilocyte, in cells that are appropriately differentiated. E5 also seems to be involved in cell transformation. E5 encodes a small protein that seems to bind to a variety of host membrane proteins including growth factor receptors. It also contains 3′ regulatory and polyadenylation sequences for all of the E region genes. Because expression of E5 is often lost during viral integration, its role in human carcinogenesis is controversial. L2 and L1 encode the minor and major viral capsid proteins, respectively. The expression of these proteins and their messages is also tightly regulated in a cell differentiation-dependent manner.

As noted above, there are more than 100 HPV types. Given the absence of serologic reagents or viral culture systems, these viruses are classified not by serotype, but by genotype. Today, a new HPV type is defined when sequences in selected genomic regions have more than 10% divergence compared to any of the known HPV types. From these definitions and from computerized analysis of these sequences, it is clear that the papillomaviruses have had a long evolution, probably coevolving with humans as well as vertebrates in general (16–19). The different viral types are not the product of simple point mutation. The grouping of papillomaviruses that is derived from sequence analysis remarkably predicts the recognized clinical groupings (20). Broadly speaking, there are cutaneous and mucosotropic groups. In the cutaneous group, there are HPVs common to the general popualtion, such as HPV-1, which is the agent of plantar warts, HPV-2 and -4, which cause common warts, and then there is a large group of 20 or more HPV types that are associated with the rare disease epidermodysplasia verruciformis (EV). Interestingly, most of the EV warts that progress to cancer are associated with HPV-5 and -8, i.e., there is a "high-risk" subgroup analogous to the subgroups recognized in the mucosotropic HPVs. In the mucosotropic group, the viruses may be broadly classified into those with a low risk of lesion progression to cancer vs. those with a moderate-to-high risk. Viruses classified as low risk are defined by the fact that they are almost never found in invasive cancers. In contrast, high-risk viruses are those that are most often found in invasive cancer. However, high-risk virus infection does not equate to the inevitable development of cancer. The molecular epidemiology of most of the moderate- or intermediate-risk viruses seems incompletely developed because of a relative lack of probes and the recent description of members of this group.

The four mucosotropic viruses, HPV-6, -11, -16, and -18, form the prototypes for the low- vs. high-risk groupings and together account for approximately two-thirds of the HPV-associated anogenital neoplasms (21). Type 6 and 11 primarily cause benign exophytic genital warts or condylomata acuminata. These are the viruses present in more than 90% of condylomas with about two-thirds caused by HPV-6 and one-third by HPV-11. They are also associated with low-grade squamous intraepithelial lesions (LSIL) and are only rarely associated with high-grade squamous intraepithelial lesions (HSIL) or invasive squamous cancers. Related viruses that produce a similar spectrum in the cervix are HPV-42, -43, -44, as well as -26, -53, -54, -55, -62 and -66. In contrast, HPV-16 is the most prevalent virus to infect the uterine cervix, is closely associated with the entire range of intraepithelial and invasive squamous neoplasia, as well as less commonly, cervical glandular neoplasia. The moderate-to-high risk types most closely related to HPV-16 include types -31, -33, -35, -52, -58, and -67. HPV-18 is the other cancer-associated prototype, which is also most commonly associated with nonsquamous cervical neoplasms. The viruses most closely related to type 18 include types 45, 59, as well as types 39 and 68. Other types such as 51 and 56 seem to have some association with cervical cancer but are genetically also related to the cutaneous group. Morphologically similar lesions at different mucosal sites are largely caused by the same mucosal viruses. Thus, laryngeal and conjunctival papillomas, which are pathologically and biologically equivalent to a condyloma, are most often caused by HPV-11 and -6. In contrast, the bowenoid dysplasias of the vulva, penis, anus, and oral cavity are most often associated with HPV-16. As will become clear, all HPV types, even the high-risk viruses, must induce the pathologic equivalent of a wart, condyloma, or LSIL, for this is pathology that supports viral replication and virion production.

HPV EPIDEMIOLOGY

Numerous epidemiologic studies have linked cervical cancer to sexual behavior (13,14,22–26). In most studies, the finding that either female promiscuity or being a monogamous female partner of a promiscuous husband confers increased risk for cervical cancer supports the concept of a sexually transmitted agent. The strongest epidemiologic risk factor is the number of sexual partners. Other behavioral correlates such as young age at first intercourse and early age of parity also seem to confer risk, although the altered hormonal environment of adolescence and its effect on the cervical epithelium may also be a risk factor. In most epidemiologic studies, cigarette smoking remains a risk factor even after controlling for sexual factors. Morphoepidemiologic studies demonstrate that the precursors of cervical cancer precede invasive cancer, with LSIL having the highest prevalence in patients in their early twenties, HSIL in the late twenties and early thirties, and invasive cancer in women ages 40 to 50. In cohorts that are more recent, there is a trend toward earlier age for each of these stages.

The epidemiology of genital HPV infection clearly accounts for the epidemiology of cervical neoplasia (24,26). Modern molecular epidemiologic analyses, as well as our understanding of the molecular biology of the virus-host cell interaction, provide a mechanistic basis for this link. The confusion in the literature of the 1980s that failed to show a strong association between HPV and cervical neoplasia was most likely due to a lack of high-quality molecular tests for HPV DNA. More recent analyses using validated methods confer relative risks for cervical neoplasia based on the presence of HPV on the order of 10 to 100 to one. These are at least an order of magnitude greater than any other epidemiologic risk factor for cancer ever described. In multivariate analyses, controlling for the presence of HPV infection leads to "dropout" of virtually all other risk factors. Thus, HPV infection confers 85 to 90% of the attributable risk for the development of cervical dysplasia. Cohort studies have shown that in cytologically normal women HPV infection precedes the development of dysplasias and that infection with HPV-16 or -18 confers the highest risk for dysplasia, particularly high-grade dysplasia. For example, in a study of 241 cytologically normal women recruited in a sexually transmitted disease clinic, the cumulative incidence of HSIL at 2 years was 28% in HPV-positive women compared to 3% in HPV-negative women (27). In a study of more than 200 "atypical" Pap smears that were reread by five expert cytopathologists, it was shown that the HPV prevalence increased from 21% in smears classified as normal to 100% in smears unanimously classified as SIL (28).

The prevalence of HPV in a population varies with the population and the method of HPV detection (29). Tests using highly sensitive amplification methods capable of detecting many viral types demonstrated the highest prevalence. When a cohort of college women were studied using L1 consensus primer polymerase chain reaction (PCR) in parallel with the commercially available Vira-Pap dot blots which only had probes for seven viral types, the HPV prevalence was 46% for the PCR assay vs. 11% for the dot blot (30,31). A conservative estimate of genital HPV prevalence in the general U.S. population is probably on the order of 15 to 20%, with serial sam-

pling over time leading to even higher estimates of prevalence. However, these rates vary with age, suggesting that patients may also clear their infection over time (32,33). Of course, this parallels cytologic observations that most cervical abnormalities regress, with only rare cases progressing to HSIL and even more rarely to invasive cancer (34–39). The overwhelming clinical problem is trying to decide which cases of low-grade morphology will or will not progress so as to more effectively direct therapy to those who need it.

HPV PATHOLOGY

The pathology of papillomavirus-associated neoplasia describes the neoplastic pathology of the cervix. In 1956, Koss coined the term koilocytotic atypia (KA) to insightfully describe cells derived from flat "warty lesions" of the cervix (40). KA is often thought to be pathognomonic for HPV infections, i.e., HPV cytopathic effect. It is the cytologic abnormality in which virion is most often detected, and is highly correlated with productive HPV infection. However, cytologic or histologic absence of KA does not in any way imply absence of HPV, more specifically absence of pathogenic HPV gene expression. Koilocytotic atypia, which under the Bethesda classification is considered a low-grade squamous intraepithelial lesion, is the most common definite abnormality in cytologically screened populations today, present in 1 to 4% of Papanicolaou ("Pap") smears (41,42). In 1976, Canadian and Scandinavian workers both described flat and inverted condylomas of the uterine cervix and reported that these lesions not only may be found in the spectrum of cervical intraepithelial neoplasia (CIN), but that they also were associated with the human papillomavirus (43–45). Supporting morphologic data linking HPV to cervical neoplasms came initially from studies of the distribution of HPV capsid protein or HPV virions in intraepithelial neoplasias. Using a broadly reactive group-specific antisera against the L1 protein, papillomavirus capsid antigen, the expression of which is a highly differentiation-dependent phenomenon, was found to be present in 50 to 60% of condylomas or LSILs with a decreasing frequency as cytohistologic grade increased (46–48). Similarly, if one uses transmission electron microscopy to search cell nuclei for virions, there is an inverse correlation with cytologic lesion grade for the detection of virus (49–51).

HPV MOLECULAR BIOLOGY

Data derived from molecular biologic studies of HPVs support the epidemiologic and pathologic associations. Molecular detection methods can be applied in a variety of ways. Analyses that destroy the sample to release the nucleic acids for analysis by necessity require morphologic correlation. Most commonly, a cellular sample is analyzed for the presence of HPV DNA by dot blot, Southern blot, or some analogous technique, more recently with improved sensitivity by coupling these methods to an amplification technology like the PCR (20,52–54). These types of studies have been strongly complemented by many direct analyses, which have used *in situ* hybridization to directly demonstrate the presence of HPV DNA or messenger RNA in defined groups of pathologies (55,56).

There are more than two dozen mucotropic HPVs that infect the genital tract. All can be found in low-grade SILs or in samples from cytologically "normal" women, and although no single type predominates, the HPV-16-related groups are the most common viruses in the cervix (20,57). The prevalence of HPV DNA in LSILs is in excess of 90%, and the same may be said for HSIL and invasive squamous carcinoma of the cervix. However, the type spectrum in the high-grade lesions is much more restricted, with just four HPV types (16, 18, 31, and 45) accounting for almost 80% of the invasive cancers. Squamous cell carcinomas account for only 80% of cervix cancers, the remainder being made up of primarily endocervical adenocarcinomas and a small number of small cell neuroendocrine carcinoma (58). Studies using sensitive methods to analyze these nonsquamous cancers and their precursors also demonstrate a very high prevalence of HPV DNA (59–62). The virus most closely related to progressive cervical squamous neoplasia is HPV-16 (63). Although accounting for fewer cervical infections, HPV-18 is more consistently associated with adenocarcinomas and small cell neuroendocrine cancers of the cervix and less frequently with invasive squamous cancer. The absolute prevalence of some of the more recently described types (e.g., HPV-31, -33, -35, -39, -42, -43, -44, -45, -51, -52, -56, and others) may be underestimated, as they have not been generally available for large-scale screening (64). Thus, the best available evidence suggests that HPV genetic material is present in more than 90% of premalignant and malignant squamous lesions of the uterine cervix. A corollary of this is that studies reporting lower association rates may be technically deficient and have a significant false negative rate (*vida infra*). The association of HPVs with squamous cell carcinomas at other body sites (vagina, vulva, anus, penis, larynx, and skin) and in a variety of genetic or induced immunodeficiency states is also well recognized (65–68).

Unlike any other candidate cervical cancer agent, HPV

DNA is not only present in every pathology linked to HPV, but also HPV messenger RNA is expressed in these lesions (69,70). The presence of viral RNA and protein expression leads to a rational framework implicating the virus in lesion pathogenesis. Patterns of viral mRNA expression vary with morphology in a tightly regulated and differentiation-dependent manner (55,69,71,72). In low-grade lesions, all viral genes are expressed as a manifestation of vegetative viral replication. In contrast, in HSIL and invasive cancer, there is a restricted pattern of viral gene expression, and E6 and E7 predominate. Cervical carcinoma cell lines such as HeLa, SiHa, and CaSki have also been found to harbor integrated HPV-16 or -18 DNAs from which the transforming E6 and E7 regions are actively transcribed (73–78).

Active transcription of HPV DNA within lesions establishes a strong molecular association of HPV with cervical neoplasia. *In vitro* cell transformation experiments additionally point to an active role for these viruses in carcinogenesis. DNA from high-risk HPV types like HPV-16, -18, -31, and -33, but intriguingly, not HPV-6 or -11, are capable of transforming epithelial cell lines in cooperation with an activated cellular oncogene such as Ha-ras, thus mirroring general concepts of multistep carcinogenesis (79–83). HPV-16 DNA alone can immortalize cultured primary foreskin keratinocytes or primary cervical cells in culture (84–87). While not inhibiting stratification of keratinocytes cultured on collagen rafts, HPV-16 can prevent cellular differentiation, thereby inducing in these artificial epithelia morphologic features that mimic CIN (88,89). Deletion experiments clarified that the essential part of the viral genome for these effects is the expression of the E6 and/or E7 region. It is also noteworthy that in these systems the transformed phenotype is not apparent until the cells have been passed through many generations, mimicking the long progression times seen in naturally occurring clinical lesions and suggesting the need for additional genetic events to manifest a high-grade lesion.

There is also an association between the physical state of HPV DNA within the cell and the malignant potential of the associated epithelial proliferation (90–95). In benign HPV-infected lesions, the viral DNAs exist as extrachromasomal plasmids, mostly as monomeric circular molecules (96). However, in most cancers, HPV DNAs are integrated into host chromosomes. Viral integration most frequently disrupts the E2 ORF, which encodes the transcription regulatory proteins. Loss of these regulatory proteins is thought to be the basis for potential dysregulation of the expression of the transforming E6 and E7 ORFs (97).

Concurrent with the revelation of HPV biology, there has been an explosion of information about the roles of cellular oncogenes in carcinogenesis (98–101). Several classes of oncogenes, including growth factors, growth factor receptors, GTP binding proteins, protein kinases, and DNA binding proteins, have been shown to be relevant to the control of cell growth. C-myc and c-Ha-ras amplification can be documented in some cervical cancers and correlates with advanced clinical stage at the time of analysis (102–105). In cervical cancer cell lines, HPV integration sites were found to be in the same general region as some of the known oncogenes, including c-myc, suggesting the possibility of transcriptional activation by the virus, although the latter has not been directly documented (106,107). In other cases, HPV DNA integrates near fragile sites (108). The significance of these observations is not clear, but again suggests the potential for multiple genetic/chromosomal events in neoplastic progression.

Observations on oncogene effects have been hard to directly relate to pathogenesis. In contrast, elucidation of the interaction of HPVs with tumor suppresser genes has been highly informative. Fusion of HPV-18-expressing HeLa cells with normal human fibroblasts or keratinocytes results in the repression of the malignant phenotype of the HeLa cell (109). Upon transplantation into nude mice, the loss of chromosome 11 from the hybrid cells results in the reversion to malignant phenotype, suggesting another tumor suppressor gene at this site. This experiment was extended by Schwarz and coworkers, who proposed that the ability of a cellular product to suppress the expression of the HPV-18 oncogene requires a humoral factor (68,110,111). Clearly, several gene products may interact to elicit or inhibit cell transformation. The human retinoblastoma (RB) gene was the first tumor suppressor gene to be characterized (112). RB either is completely absent or has significant deletions in tumors from patients with retinoblastoma, breast cancer, and in several other epithelial tumors such as squamous cell carcinoma of the head and neck (113–116). The transforming E7 protein of HPV-16 has structural and functional similarities to the E1A antigen of adenovirus, the large T antigen of SV40, and the host cellular protein cyclin D1 (117–122). All of these proteins have the ability to form inactivating complexes with the retinoblastoma antioncoprotein by competitive binding to the "RB pocket." This functional inactivation causes the release of a potent host transcription factor, termed E2F, which is capable of activating transcription of a variety of host genes, many of which are involved in DNA synthesis and cell cycle progression. Similar complexing and inactivation of the p53 suppressor gene by the E6 proteins of

high-risk viruses like HPV-16 has also been demonstrated (123–129). E6 binds to p53 via an E6-associated host protein. This binding promotes the ubiquitin-dependent degradation of p53, the functional equivalent of mutational inactivation. p53 is a prime regulator of cell proliferation via transcriptional transactivation. For instance, p53 activates transcription of p21 (also called waf 1 or cip 1), a potent inhibitor of cyclin-dependent kinase. Therefore, either mutation or E6-mediated degradation of p53 can lead to derepression of cell cycle regulation. In rare instances in which a cervical cancer has been shown to not contain HPV, p53 mutation has been found, whereas mutation is absent in the usual case (130–133). Interestingly, the E6 proteins from low-risk HPVs are incapable of causing this degradation. Therefore, E6 undoubtedly has other roles in the virus-host interaction other than p53 inactivation, such as direct effects as a transcription factor (134).

HPV-MEDIATED CARCINOGENESIS

Taken together, the above data have led to a molecular model for HPV-induced carcinogenesis. This model involves the interaction of HPV gene products with what is recognized to be a tightly controlled network of cellular oncogenes and antioncogenes, which control cell proliferation and DNA synthesis. Histogenetically, papillomaviruses must infect the "reserve, basal, or stem" cell population of the cervical transformation zone, cells that have the potential to differentiate along squamous, glandular, or neuroendocrine lines and are responsible for epithelial maintenance. In cells committed to squamous differentiation, there is an orderly program of maturation throughout the epithelial thickness, both at the morphologic and molecular level. In normal squamous differentiation, the only cells capable of cell division are the basal or parabasal cells. In morphologically normal, but HPV infected, basal cells, papillomavirus gene expression is inhibited to essentially maintenance levels. Productive HPV gene expression is tightly regulated and permitted only in cells that have begun squamous maturation, with concurrent loss of proliferative capacity (55,71,72,135–138). In the immediate suprabasal zone, there is expression of the early regions of the virus, and as the cells differentiate, there is an induction of all viral genes, as well as viral DNA synthesis, leading to assembly and production virions in the cells just beneath the surface. In the cervix, we recognize such lesions as low-grade squamous intraepithelial lesions or mild dysplasias, most of which at some point demonstrate koilocytotic atypia. Such lesions usually regress or maintain themselves for extended periods. An explanation of some of the diagnostic criteria used by pathologists is implicit in this program of differentiation-linked expression. The nuclear enlargement and hyperchromasia recognized as atypia is a direct result of E6/E7-mediated activation of host DNA synthesis. In a low-grade lesion, this is regulated to occur in cells that can no longer divide, i.e., the intermediate squamous cells, and is primarily directed at the production of viral DNA (138). Given the small size of the viral genome, the several thousand copies of the virus present in a productively infected cell clearly cannot account for the two- to fourfold nuclear enlargement that is observed. It is a diagnostically fortunate coincidence that ineffective (in the sense of cell division) E6/E7-mediated host DNA synthesis produces the enlarged nuclei and increased N:C ratio that one recognizes as abnormal. If the process is not fully developed or is perhaps regressing, then the cells derived from the surface often have less nuclear abnormality (? atypical squamous cells of uncertain significance [ASCUS]) than seen in classical dysplasia. In contrast, in the fully developed case, they are classified as being derived from a mild dysplasia/LSIL. If the cells also have the correct amount and form of the cytokeratin-binding protein HPV E4 expressed, then they appear as koilocytes. Koilocytotic atypia, while very often present, does not have to be seen to recognize a low-grade lesion. Every cytologist recognizes cells derived from the upper levels of a mild dysplasia that meet the diagnostic criteria for dysplasia, yet do not have the characteristic perinuclear halo termed koilocytosis. Such lesions are just as HPV-associated as those that do have koilocytes, and the differences undoubtedly represent temporal variation within the life cycle of a low-grade lesion.

If viral gene expression is so tightly regulated, how do high-grade lesions develop? The *sine qua non* of high-grade dysplasia is morphologic evidence of basal-like cell proliferation. In these cells, the coordinate link between differentiation and viral early gene expression is lost. How this occurs is unclear, although it certainly must be a rare event(s) given the relative frequency of low vs. high-grade lesions. Potential mechanisms might include viral integration or mutations in HPV E2, such that E2-controlled regulation of E6/E7 expression is lost. In such cases, the viral oncogenes E6 and E7 are inappropriately expressed in a population of cells that retain the capacity to divide, thereby initiating cell proliferation. As this population of cells proliferates, it overtakes the epithelium, producing lesions that are by definition characterized by less orderly squamous maturation and basal-like cell overgrowth. Possible promoters of this process could be smoking, other viruses, random mutation, etc. The relative infrequency of these effects is bio-

logically manifest by the latency and relative rarity of HSILs vs. LSILs. Progression to the proliferative phenotype occurs most frequently, albeit not exclusively, with high-risk viral types, and results in the high-grade squamous intraepithelial lesions also called moderate squamous dysplasia, severe squamous dysplasia, or squamous carcinoma *in situ*. Thus, the Bethesda system's break between low-grade vs. high-grade follows in part from the biologic changes manifest between these morphologies. Indeed, from the standpoint of epithelial biology, there is little rationale for separating moderate from severe dysplasia in that the critical break occurs between mild and moderate dysplasia, with the switch to a proliferative as opposed to a differentiated phenotype.

In high-grade squamous intraepithelial lesions, the proliferating basaloid cells, driven by E6/E7 overexpression, are at much greater risk for the acquisition of additional genetic errors, clonal selection, etc., perhaps under the influence of the same external mutagens and/or host genetic predisposition, which further promotes the development of the fully malignant phenotype, most often an invasive squamous cell carcinoma. The different subtypes of squamous cancer are probably related to the multistep and somewhat random nature of the process. The proportion of different types just reflects the relative likelihood of different genetic pathways to a "successful" cancer, in part modulated by the microenvironment in which the lesion develops. Hence, early observations that keratinizing cancers are often more ectocervical than large cell-nonkeratinizing or small cell malignancies, which tend to originate higher in the endocervical canal, have some contemporary validation.

Given this model for cervical squamous neoplasia, one still needs to account for glandular and small cell neuroendocrine neoplasms. Of course, reserve cells that are already committed to glandular differentiation are, because of a lack of an appropriate differentiation environment, not going to be productive of virions. The productive viral life cycle requires the cellular milieu of orderly squamous differentiation. If this is true, then viral infection in cells committed to glandular differentiation most often results (from the viral standpoint) in an abortive or latent infection of morphologically normal endocervical cells. Rarely, dysregulation of viral early gene expression occurs in these usually nonpermissive cells. This leads to hyperproliferative lesions of glandular cells, which pathologists recognize as severe endocervical dysplasia/adenocarcinoma *in situ* (AIS). There is no biologic correlate in this model of a low-grade glandular dysplasia. Hence, this explains the inability of pathologists to reproducibly recognize, either cytologically or histologically, a clinically meaningful lesion less severe than what most call AIS. HPV-18 seems to be more successful at inducing this in glandular cells than HPV-16. Perhaps this is because HPV-18 has a greater disposition to integrate into the genome and perhaps because it may have some preference for cells predisposed to other than squamous differentiation. Parenthetically, little if anything is known about the mechanism of HPV-type-specific cellular tropism. However, no HPV type can be exclusively trophic for nonsquamous cells, because if this were so, that strain of virus would be eliminated from the population. Depending on the genetic switches that over time accompany virally induced glandular proliferations, the outcome may be an invasive adenocarcinoma, most often endocervical, but less frequently of another type, e.g., endometrioid, clear cell, etc. The relative frequencies of the different types of cervical adenocarcinomas again may just reflect the relative frequency of the different populations committed toward various types of differentiation. Essentially identical arguments may be made for the development of small cell neuroendocrine carcinomas, tumors that are almost always associated with HPV-18 and whose low incidence probably reflects the relative abundance of a susceptible neuroendocrine-committed precursor cell population and the rarity of "successful" viral induction of cell proliferation in such cells. None of the above precludes alternative pathways of carcinogenesis unrelated to HPV (105,138,139). However, in the uterine cervix, the ubiquity of HPV infection is the predominant force driving neoplastic development. Fortunately, when compared to the high prevalence of the virus, progression is an extremely rare occurrence.

HPV SCREENING

As noted earlier, the estimated overall HPV prevalence in the U.S. target population is approximately 20%. The prevalence varies greatly with age. For female ages 20 to 29, the prevalence is probably 40 to 50%, and this decreases by 50% for each decade of age until a background level of around 5% is reached. These data have implications for a brief discussion about the utility of human papillomavirus testing as a screening procedure.

By now it should be clear that virtually all lesions encompassed by the term "cervical neoplasia" are HPV-associated. The epidemiologic and molecular evidence supporting this finding has been presented and is hopefully convincing. Furthermore, virtually 100% of invasive carcinomas from around the world have been shown to be associated with a limited spectrum of HPV types (135,140–142).

Given the strength of these associations, an obvious question is whether screening for HPV using some sort

of molecular diagnostic would be superior for selecting the population at risk for cancer development (143–147). The answer to this apparently simple question is unfortunately complex. Part of the problem is technical. Which HPV test should be used? HPV testing, as all molecular diagnostics, is continually evolving (144,145,148–152). Until recently, there has been only one commercially available FDA approved test for HPVs, the Hybrid Capture Tube test (HCT) marketed by Digene Diagnostics (Gaithersburg, MD). The sensitivity, specificity, and predictive values of the "tube" test with a 10 pg/ml cutoff value for a group of 11 to 13 high-risk viruses have been well-characterized (152–154). Compared to PCR analysis using L1 consensus primers, the HCT has a lower sensitivity. However, the HCT is more specific for the presence of clinically detectable cervical abnormalities compared with PCR, which because of its higher sensitivity, picks up a significantly higher population of patients without clinically detectable disease.

As noted above, molecular technologies continue to evolve. The newest version of the Hybrid Capture test (HC II, approved in March 1999) is relatively semiautomated, uses a microtiter format, and has up to 50 times the analytic sensitivity of the current test. Whether the improved sensitivity is of clinical benefit greatly depends on whether one is using the test for screening vs. diagnosis/triage and the population characteristics. The interplay between sensitivity, specificity, and disease prevalence needs to be considered when evaluating the utility of any test. Likewise, PCR/amplification technologies are rapidly evolving. In addition, the expanding sequence database of all relevant human papillomaviruses makes it likely that the new powerful "DNA-chip" technologies may possibly replace or augment current HPV testing methods.

Might HPV testing be a better screening method? This question has been most thoroughly examined by workers in the Netherlands who have proposed using an extremely sensitive PCR-based method as the first step in a cervical cancer screening program (155–157). If one were designing a cervical cancer screening program from scratch, this approach makes a tremendous amount of sense. Nearly 100% of the pathology of interest is HPV-positive. Conversely, if after using a sufficiently sensitive screening test an individual were not HPV-positive, the incidence of disease would be so low as to make screening nearly worthless. Combining the high prevalence of human papillomaviruses in the pathology of interest with the relatively long time frame from acquisition of infection until the development of the target, cervical cancer, immediately brings the relative value of initial triage based on HPV status into focus. The lower the prevalence of HPV in the population to be screened, the better the performance profile of an extremely sensitive HPV screening test. For instance, the incidence of cervical cancer in women under 25 to 30 years of age is extremely low, and the prevalence of HPV in the United States drops from approximately 40% at age 20 to 10 to 20% at ages 30 to 40 (or as low as 4 or 5% at age 30, as it is in the Netherlands). Under these conditions, it may not make sense to spend resources on screening young women, most of whom develop only transient, low-grade lesions. The Dutch proposal seeks to screen the entire population at age 30 with the most sensitive available HPV test, combined with a single cytologic screening. Patients who are positive on either test would be entered into a program of more intense routine screening, whereas the "double negative" patients would be returned to the general population pool that would be then screened on the long-interval basis of 5 to 10 years. Again, if the prevalence of detectable virus is low and the disease prevalence is also low, such a system makes for extremely rational triage and resource utilization. The arguments become even stronger if the cost and reliability of the HPV test becomes comparable to cytologic methods. Indeed, in some recent studies, HPV testing seems more reliable than the Pap smear due to superior sensitivity in identifying patients with cervical abnormalities. For instance, a large, recently published triage study of ASCUS patients evaluated Hybrid Capture II testing for "oncogenic" HPVs vs. repeat smear as an index for colposcopic referral (158). The sensitivity for HSIL+ in the HPV testing arm was 89.2% with a specificity of 64.1%. In contrast, the sensitivity for repeat Pap smear was 76.2%. This difference approached statistical significance. This and other studies strongly suggest that HPV testing will evolve into routine clinical practice. Furthermore, prospective studies addressing a rational basis for HPV primary screening are needed and planned in the Netherlands and at other sites. Whether such a program could be tested in the United States is debatable given the relative mobility of the U.S. population and the strongly ingrained emphasis on annual Pap smear screening.

HPV VACCINES

The recognition that human papillomaviruses are the primary etiologic agent for cervical cancer strongly raises the possibility of the use of HPV vaccines both for the potential treatment as well as prophylaxis of cervical cancer (23,159–167). A successful prophylactic HPV vaccine could virtually eliminate the need for cervical cancer screening programs. This admirable long-term goal is just possibly coming into reach.

The multiplicity of viral types in the cervix is a problem for vaccine development. It is unclear whether immunity to any specific type provides cross-reactive immunity to other types. Thus, the ultimate vaccine may likely be a complex polyvalent mixture. Until recently, the lack of an abundant source of HPV antigens has markedly impeded vaccine development. However, recombinant methods capable of generating virus-like particles containing the HPV L1 and L2 capsid proteins have been the major technical advance promoting HPV vaccine development. Studies performed in animals reveal consistent and promising findings for the development of a prophylactic HPV vaccine. Vaccines developed in rabbits, cows, and dogs all show great promise. Canine oral papillomaviruses (COPVs) are effectively prevented by intradermal injection in the footpad of either a formalin-inactivated COPV wart extract or COPV L1 virus-like particles. Immunization of approximately 60,000 beagles over a 3-year period resulted in complete protection against naturally acquired COPV-induced warts.

Approximately 80% of HPV cancers are associated with a limited type spectrum of HPV-16, -18, -31, and -45. Several vaccine trials, most initially targeting HPV-16, are undergoing Phase I and Phase II testing. Obviously, the long natural history of both HPV infection and cervical cancer, together with the fact that the optimal target population involves young people, before the onset of sexual activity, complicates the development of such vaccines. However, the potential success of an HPV vaccine program could produce the first example of true cancer prophylaxis, and ultimately lead to the elimination of the entire cervical cytology screening system.

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